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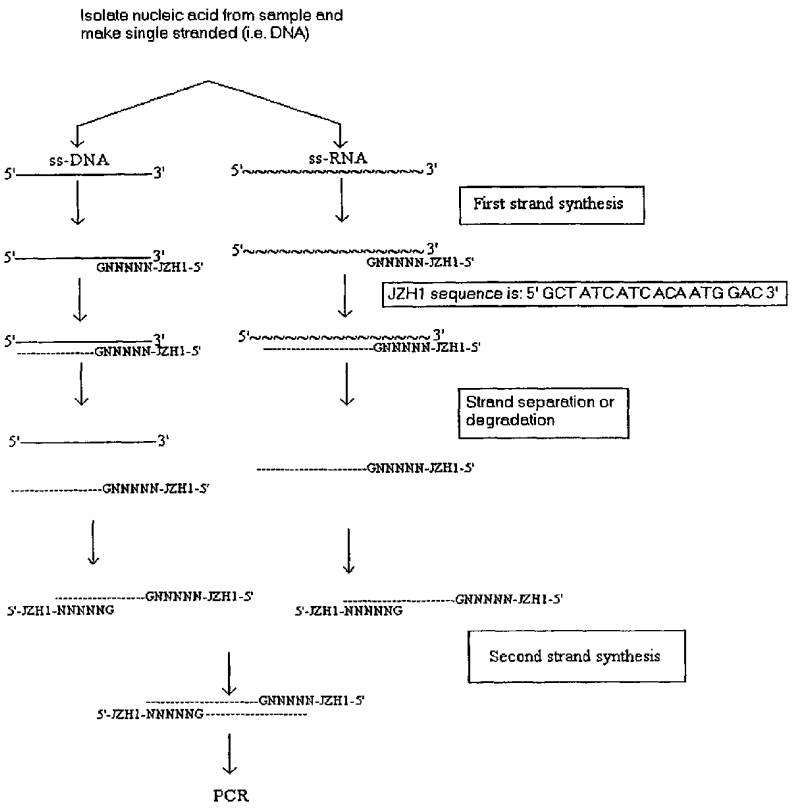

(57) Abstract: The invention provides in one embodiment a method for amplifying nucleic acid in a sample comprising providing said sample with a set of primers to enable synthesizing at least one nucleic acid strand complementary to at least part of said nucleic acid, wherein said set of primers comprises between 3-8 random bases preferably clustered near the 3' end of each primer in said set of primers. In a preferred embodiment the method is used for determining whether a sample, derived from a patient, mammal, poultry and fish, comprises nucleic acid of a pathogen. The method is further suited for the typing of said pathogen and the typing of a particular variant of said pathogen. The method is also suited for the elucidation of the gene-expression profile or genetic profile of cells.

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A universal nucleic acid amplification system for nucleic acids in a sample

The invention lies in the field of diagnostics. More in particular the invention lies in the field of molecular diagnostics.

The increased knowledge of the molecular basis for disease, has generated an increasing demand for more and more sophisticated diagnostic methods that can help identifying the exact molecular cause of the disease. In particular for infectious diseases, the clinicians want to be able to rapidly identify the pathogen. Importantly, concurrent accurate typing and discrimination of different strains of a pathogen is desired. For instance in cases where certain strains have a particular unfavorable phenotype. For instance in the case where the pathogen is capable of rapid mutation of its genome to counteract selective pressures induced by the patient and/or the treatment. One non-limiting example of such a pathogen is of course Human immuno deficiency virus (HIV). HIV is, for instance, capable of evading selective pressure induced by nucleotide analogues through mutation of the reverse transcriptase enzyme. To be able to predict which nucleotide analogue, if any, would benefit the patient, it is desired to know in advance, i.e. before treatment starts, which genotype(s) of HIV prevail in the patient.

One possibility to find the pathogen causing the disease is to harvest a sample from the patient comprising the pathogen and culturing the pathogen on suitable media in case of a bacterial pathogen or a suitable marker cell line for a viral and/or mycobacterium pathogen. Following and/or during the culture of the pathogen it may be typed. This culture process can be combined with for instance antibiotics and/or other medicines to find the relative resistance/sensitivity of the pathogen to said medicine. This so-called culture driven testing has several advantages and is indeed routinely applied for a number of diseases.

However, generally, the considerable amount of time involved with the culture process necessitates that a treatment schedule is already started prior to the identification of the causative agent. This is not desired since the treatment started may prove to be ineffective. Moreover, for many pathogens a culture system is as yet not available. Another problem with the culture system is the inherent variability of the procedure. Not all pathogens are equally well cultured outside the body of a patient. In addition, since viability of the pathogen is essential, differences in the handling of the sample outside the body will result in variability of the result. Moreover, the costs involved with the initiation of a screen for a wide variety of different possible causative agents in any clinical sample is with the culture system considerable.

For this reason there is a need for a rapid system for the typing of a pathogen that is versatile, reliable and at least in part able to discriminate between different variants of pathogen. A number of different strategies have been tried. One such strategy relies on the detection of pathogen derived nucleic acid in a sample. To be able to rapidly detect such nucleic acid, a nucleic acid amplification step is usually required.

Many nucleic acid amplification methods have been devised that are able to specifically detect a certain pathogen and possibly even a number of different strains of said pathogen. However, such methods usually require the clinician to have at least a first idea of the kind of pathogen that may cause the disease in the patient. This is frequently not the case.

The present invention provides a method for detecting, quantifying and/or simultaneous typing of a variety of different nucleic acid sequences in a sample. Preferably said nucleic acid sequences comprise nucleic acid from a micro-organism and/or derivative thereof. A micro-organism can be a bacterium, a phage and/or a virus.

Preferably, said micro-organism is a pathogenic micro-organism. The present invention further allows a discrimination between different strains of micro-organism or other sequences. The present invention is not only useful for the typing of a pathogen in a sample of a patient but is also applicable for the typing of a pathogen in a sample derived from an animal. Preferably, said animal has a commercial and/or emotional value to a human, such as a pet, a farm animal and/or an animal living in a natural reserve. The invention is also suitable for application in poultry and fish. The method of the invention is of course not only suited for the typing and/or detection of a pathogen. The method is generally suited for the typing and/or detection of nucleic acid in a sample. For instance in case of cellular DNA or RNA, the method can be used for creating a genetic, respectively, expression profile of the nucleic acid in the sample. Knowing the origin of the nucleic acid in the sample then allows the correlation of the profile with the origin. In case the origin is nucleic acid of (a specific part of) an individual, the profile, or a part thereof, can be correlated to for instance a database of profiles, or parts thereof, of other individuals. Matching of the profile(part) to known profiles(parts) allows the correlation of the profile(part) of the individual with the phenotypes of individuals with matching profiles(parts) displayed by these other individuals. Thus the method of the invention can be used generally for the typing and/or detection of nucleic acid in a sample.

In one aspect the invention provides a method for amplifying nucleic acid in a sample comprising providing said sample with a set of primers comprising between 3-8 random bases and at least 8 essentially non-random bases, subjecting said sample to a first nucleic acid amplification reaction, providing said sample with at least one second primer comprising at least 8 bases essentially identical to said non-random bases, subjecting said sample to a second

amplification reaction and detecting nucleic acid amplified in said sample. Typically only a limited amplification of nucleic acid will occur in said first nucleic acid amplification reaction. Said first and second amplification reaction are preferably performed separately, optionally including a step to remove any unused primer in said first amplification reaction. This way the reproducibility of the method is best controlled. However, the first and the second amplification reaction may also be performed simultaneously.

Nucleic acid in said sample may be DNA and/or RNA. A double stranded nucleic acid can be denatured into essentially single stranded nucleic acid prior to the priming of synthesis of a complementary strand of nucleic acid. The complementary strand may be DNA and/or RNA. Synthesis of said complementary nucleic acid is performed under conditions and using enzymes which are known in the art, such as for instance conditions and enzymes commonly used for polymerase chain reaction and/or NASBA.

The number of nucleic acids amplified with the method of the invention is dependent on the amount and on the complexity of the nucleic acid in the sample. When the complexity, i.e. the number of different sequences in the nucleic acid(s) is low, a small number of nucleic acids will be amplified with the method of the invention. In this case some nucleic acids will be dominant in the amplificate resulting a banding pattern when the amplificate is run on a gel. On the contrary when the complexity of the nucleic acid in the sample is high, many nucleic acids will be amplified resulting in a smear when the amplificate is run on a gel. An example of nucleic acid with a particularly low complexity is nucleic acid derived from a small virus and/or plasmid (typically smaller than 10 kb). An example of nucleic acid with a particularly high complexity is cellular DNA (typically comprising > 10^8 kb). It is clear that the mentioned examples are non-limiting. Many different complexities are possible and also mixtures of low and high complexity nucleic acid can be used for the present

invention. However, samples comprising only nucleic acid of low complexity that is smaller than 1 kb. are not suited for the present invention. As mentioned above, the number of nucleic acids effectively amplified with the method of the invention is also dependent on the amount of nucleic acid in the sample. When the sample comprises particularly low amounts of nucleic acid some nucleic acids will be dominant in the amplificate resulting a banding pattern when the amplificate is run on a gel. On the other hand when the amount of nucleic acid in the sample is high, many nucleic acids will be amplified resulting in a smear when the amplificate is run on a gel.

The dependencies on the complexity and the amount of nucleic acid in the sample are intertwined. For example, when the complexity of the nucleic acid in the sample is high but the amount of nucleic acid in the sample is low, some nucleic acids will be dominant in the amplificate. As will be discussed in more detail later in the description amplificates comprising dominant nucleic acids and amplificates comprising many different nucleic acids and amplificates comprising both dominant and many different nucleic acids are useful in the present invention. However, the present invention is only useful for when at least two nucleic acids are amplified. Preferably, at least 5 nucleic acids are amplified. More preferably, at least 50 nucleic acids are amplified. Typically, the method is used to amplify approximately 10.000 different nucleic acids, for instance in samples comprising a relatively large amount of complex nucleic acid.

Although the dependencies mentioned above are intertwined, the person skilled in the art will be able to determine what amount of nucleic acid is required to obtain a specific resulting amplificate.

Preferably, said sample is provided with a set of primers comprising at least three or more different primers. Preferably said set comprises at least ten different primers.

A nucleotide may be an A, T, C, G, or a U and/or a functional equivalent thereof. A functional equivalent of a nucleotide is any substance capable of mimicking at least in part an A, T, C, G, U in a nucleic acid. For instance, known nucleotide analogues are suited substances. Also substances which can mimic a couple of nucleotides such as for instance inosine are suitable substances. Preferably, said nucleotide analogues allow continued synthesis of the nascent strand. With the term random base is meant that between any two primers in said set of primers, there is at least one nucleotide or a functional equivalent thereof, at a certain position, that is different between the two primers.

Apart from the random bases, primers in said set of primers further comprise an essentially non-random number of bases. This has the advantage that for subsequent amplification and/or detection of synthesized nucleic acid an essentially known template is provided such that one or more new primers can be devised that can be used for the subsequent amplification and or detection of said synthesized nucleic acid. Preferably said essentially non-random number of bases comprises between 17-22 nucleotides. Subsequent amplification is typically performed with at least one primer comprising a sequence essentially identical to a sequence formed by non-random bases in said at least one primer. There can of course be more than one second primer. Furthermore, a second primer can comprise additional nucleotides than the nucleotides required to create identity to a non-random sequence of a primer in the first set of primers. Additional nucleotides at the 3' end can be advantageously used in applications wherein additional specificity is required in the amplified product. Additional nucleotides at the 5'end can be advantageously used for the introduction of restriction enzyme sites that can be utilized to clone amplified nucleic acid. Cloning of amplified nucleic acid is often desired when amplified nucleic acid needs to be sequenced.

In the present invention the number of random bases in the set of primers has been observed to be of crucial importance to the practical application of the method of the invention, for instance for the detection, quantification and/or typing of nucleic acid in the sample. Especially, this is crucial to the detection of nucleic acid from a wide variety of different pathogens. When less then 3 random bases are used in the set of primers, the subsequent amplification is not sufficiently versatile to detect a wide variety of different nucleic acids (nucleic acid with different sequences), such as from a wide variety of different micro-organisms. Presumably due to lack of hybridization capability the various nucleic acids. When more than 8 random bases are used in the set of primers, the signal detected is too specific for particular nucleic acids. This leads, in the case when the method is used for the detection of a micro-organism, such as a pathogen, to the situation that nucleic acid of a micro-organism present in the sample, may not be detected with sufficient sensitivity. This is presumably due to the fact that not all possible combinations of 9-mers can be included in a practical way in the amount of primer that can be used in the method of the invention. Without being bound by theory it is the observation in the present invention that for the capability to detect a wide variety of different nucleic acids, it is necessary to have in the set of primers between 3 to 8 random bases. Preferably, said set of primers comprises between 4-7 random bases. More preferably said set of primers comprises 5 or 6 random bases. To increase the specificity of the reaction said random bases are preferably clustered at the 3' end of the primer. In the present invention it has been observed useful for optimal yield of amplificate to include a G at the extreme 3' end of the oligonucleotides of the set of primers. A set of primers of the invention therefore preferably comprises a G at the extreme 3' end of at least most and preferably all of the oligonucleotides contained in the set of primers.

In a preferred embodiment of the invention, the non-random bases in said set of primers comprise a sequence enabling non nucleic acid primed nucleic acid synthesis. Such a sequence may be used to obtain further amplification of complementary nucleic acid which strengthen the signal obtained from the method of the invention. Moreover, the further amplification may be used in a method for determining at least part a sequence of amplified nucleic acid such that amplified nucleic acid may be typed and/or variants of different nucleic acids such as different variants and/or strains of a micro-organism may be determined. Preferably, said non nucleic acid primed nucleic acid synthesis comprises transcription.

In a preferred embodiment said set of primers comprises the sequence,

- GCT ATC ATC ACA ATG GAC NNN NNG and/or
- AAT TCT AAT ACG ACT CAC TAT AGG GNN NNN G,

wherein N can be any nucleotide or functional equivalent thereof.

For the detection of a wide variety of different nucleic acids, such as from different micro-organisms, pathogens and/or different variants of a particular micro-organism, it is essential that the amplificate of the amplification reaction is scrutinized. This can be done through detecting the amplificate with a probe specific for amplified nucleic acid, for instance a probe specific for nucleic acid of a micro-organism such as nucleic acid from a pathogen and/or variant of said pathogen. Alternatively, the amplificate is at least in part sequenced, wherein the resolved sequence is specific for nucleic acid of said pathogen and/or variant of said pathogen.

Sequencing of at least part of the amplificate is particularly favorable when the complexity of the nucleic acid in said sample is relatively small. Particularly when said sample comprises essentially one type of nucleic acid, such as nucleic acid from one micro-organism. However,

sequencing of at least part of the amplificate is also possible when the sample comprises two, three or more types of nucleic acid. In this embodiment of the invention, however, the sample preferably does not comprise more than 5 different types of nucleic acid in a substantial amount, in this embodiment of the invention. In one embodiment of the invention it is, with this method possible to obtain a complete or at least nearly complete sequence of a particular nucleic acid present in said sample. A low complexity of the nucleic acid in the sample can be obtained in various ways. For instance in applications wherein the nucleic acid of for instance a micro-organism, preferably a virus and/or a phage is collected into an enriched fraction. For instance a sample of cell free serum obtained from an HIV infected patient will be enriched for nucleic acid of HIV viruses. Such samples or parts thereof may be used in a method of the present invention. A sequence of an HIV virus present in said sample can then be determined by sequencing of the amplificate obtained with the method of the invention. Furthermore, sequencing of the amplificate will also enable the typing of at least the dominant HIV variants in the sample. Alternatively, a sequence may be generated representing a gross average of the various variants of HIV in said sample. For this embodiment of the invention a sample comprising a low complexity of nucleic acid is preferred. A low complexity of nucleic acid in the sample does not mean that said sample may not contain complex nucleic acid such as cellular DNA. It can contain complex nucleic acid as long as the amount (in weight) of complex nucleic acid does not exceed the amount (in weight) of said low complexity nucleic acid. Preferably, the amount of complex nucleic acid does not comprise more than 25% of the nucleic acid in the sample. More preferably, the amount of complex nucleic acid does not comprise more than 10% of the nucleic acid in the sample. Of course it is clear to the person skilled in the art that this feature of the present invention is not only useful for the sequencing

and/or typing of different HIV variants but is generally applicable for the typing of nucleic acid in said sample.

In another embodiment of the invention said detecting of amplified nucleic acid comprises subjecting at least part of said amplified nucleic acid to a hybridization reaction with a multiplicity of nucleic acids preferably present in a microarray and/or DNA-chip and detecting whether amplified nucleic acid hybridized with one or more nucleic acids of said multiplicity of nucleic acids. This embodiment is particularly useful when the complexity of the nucleic acid in the sample is relatively large. This embodiment is also very useful when the type of nucleic acid present in said sample is not known. Preferably, the multiplicity of nucleic acids comprise micro-organism nucleic acid, or nucleic acid that is a reflection of nucleic acid expressed by a cell. The cell may be any type of cell. When the multiplicity of nucleic acid comprises a reflection of nucleic acid expressed by a cell, it is preferred that the nucleic acid in said sample comprises RNA that is or was expressed by a cell. In such case the RNA is preferably first transcribed into DNA, for instance with a primer capable of recognizing the poly A tail of mRNA.

The method of the invention can further comprise one or more additional amplification reactions using one or more other primers. Such an additional amplification reaction can be advantageously used to pre-amplify "certain" nucleic acid in the sample. Alternatively, an additional amplification reaction can be used to further amplify at least part of the amplificate of said first and/or the second amplification reaction. In this embodiment, therefore a method of the invention is provided, further comprising an additional nucleic acid amplification of nucleic acid in said sample using at least one primer comprising essentially non random bases.

In one embodiment the invention provides a set of oligonucleotides comprising a sequence

- GCT ATC ATC ACA ATG GAC NNN NNG and/or
- AAT TCT AAT ACG ACT CAC TAT AGG GNN NNN G,

wherein N can be any nucleotide or functional equivalent thereof. In these sets of oligonucleotides N delineates the position of a random base and C, A, T and G the position of a non random base.

In another aspect the invention provides the use of a set of oligonucleotides and/or primers of the invention for the preferred amplification of at least part of a viral nucleic acid. Preferably said set of oligonucleotides and/or primers comprises between 3 to 8 random bases clustered around the 3' end of said oligonucleotides and/or primers and an essentially constant sequence at essentially the 5' end of said oligonucleotides and/or primers for priming the synthesis of a complementary nucleic acid in a nucleic acid amplification method. Typically, said set of oligonucleotides and/or primers provide one or more essentially constant templates for detection and/or further amplification of said complementary nucleic acid. Preferably, said set of primers and/or oligonucleotides comprises a sequence

- GCT ATC ATC ACA ATG GAC NNN NNG and/or
- AAT TCT AAT ACG ACT CAC TAT AGG GNN NNN G,

wherein N can be any nucleotide or functional equivalent thereof. In these sets of oligonucleotides N delineates the position of a random base and C, A, T and G the position of a non random base. Preferably, said set of primers provide one essentially constant template for detection and/or further amplification of said complementary nucleic acid.

In yet another aspect the invention provides the use of a set of primers comprising between 3 to 8 random bases clustered around the 3' end and one or more essentially constant sequences clustered at essentially the 5' end of

each primer in said set of primers, in a nucleic acid amplification reaction comprising nucleic acid for providing complementary nucleic acid generated with said set of primers in said amplification reaction with one or more tags enabling further amplification and/or detection of said complementary nucleic acid.

In yet another aspect the invention provides a kit for the amplification of nucleic acid in a sample comprising at least one random primer comprising between 3-8 random bases. Preferably, said kit comprises at least one set of oligonucleotides and/or primers of the invention. Preferably said set of primers and/or oligonucleotides further comprise one or more essentially constant sequences clustered at essentially the 5' end of each primer in said set of primers. Preferably, said nucleic acid in a sample comprises nucleic acid from a micro-organism or a derivative thereof.

In yet another aspect the invention provides the use of a kit of the invention in a method or a use of the invention.

Nucleotide sequence analysis has become an important tool in modern molecular biology. Recent technological advancements have enable high throughput sequencing protocols that generate multiple sequences of 200-800 nucleotides in length. On the most modern systems 96 of such sequences can be determined simultaneously.

In order to make optimal use of the high throughput capabilities of the modern sequencing methods a good strategy is important. In particular sequence analysis of long stretches of nucleotides (>2 kilobase) and small sample sizes (or a combination of both) is a challenge. In one embodiment the present invention discloses a method that enables non specific amplification of nucleic acid in a sample and relative simple sequence analysis of long stretches of nucleotides.

The method consists of a number of steps that can be described as follows:

1) First strand synthesis.

The sample nucleic acid is used to copy in a first strand synthesis (i.e. cDNA synthesis) using reverse transcriptase (in case RNA or DNA is the nucleic acid being sequenced) or DNA dependent DNA polymerase (in case DNA is the nucleic acid being sequenced), see figure 1. The oligo's that are used to prime the first strand synthesis consist of a specific sequence at the 5' part of the oligo and a random sequence at the 3' part of the oligo. An example of such an oligo is JZH2R that has the following sequence:

5' GCT ATC ATC ACA ATG GAC NNN NNG 3'

Typical of JZH2R is that the oligo has a G residue at its 3' end, flanking the random sequence. The length of the cDNA will vary depending on the exact location where the random part of the oligo was hybridized and the length of the elongation by the polymerase.

2) Stand separation or degradation

Following the first strand synthesis the newly made DNA strand is separated from its template strand by denaturation or by degradation of the template strand with RNase H in case the template strand was RNA, see figure 1.

3) Second strand synthesis

Using oligo JZH2R in combination with a DNA dependent DNA polymerase a second DNA strand is synthesized complementary to the first strand synthesized (i.e. the cDNA), see figure 1.

4) PCR amplification

The newly formed double stranded DNA molecules of variable length are amplified by PCR using an oligo primer that fits the specific part of the oligo that was used in the first and second strand protocol described above. In case of JZH2R that oligo primer would be 5' GCT ATC ATC ACA ATG GAC 3', which is named JZH1, see figure 1.

5) Cloning and sequencing

The PCR fragments that display a wide variety in length are

cloned into a plasmid and used for transformation of *E. coli* cells using standard protocols known by persons skilled in the art. After growing of individual colonies overnight the plasmids of the individual colonies are isolated and used for sequence analysis of both strands of the DNA, obtaining a sequence of 400-1000 nucleotides per clone.

In addition, the above mentioned method can be used for non-biased amplification of all RNA's or DNA from a sample from a small amount to amounts sufficient for analysis of labeling.

With the incorporation of an RNA polymerase promoter sequence instead of the specific sequence part of the primers the method can be coupled to a transcription reaction. An example of such a primer would be:

5, AAT TCT AAT ACG ACT CAC TAT AGG GNN NNN G 3'

containing a T7 RNA polymerase promoter sequence. Following the PCR step (see flow chart in figure 1) a transcription reaction can be performed using T7 RNA polymerase translating the PCR fragments into RNA. Approximately 100-1000 copies of RNA are made per DNA template in a transcription reaction with T7 RNA polymerase. During the transcription reaction the RNA may be labeled enabling subsequent analysis of the RNA, for instance via hybridization on a microarray or DNA chip.

In another embodiment the method can be used for the non-biased amplification of mRNA. In that case the first strand cDNA synthesis will be performed with an oligo that consists of a poly T stretch (10 to 30 T residues) at the 3' end and a specific sequence at the 5' end (e.g. 5' 5' GCT ATC ATC ACA ATG GAC $T_{(10-30)}$ 3'). For the second strand synthesis an oligo with a random sequence can be used, e.g. JZH2R (5' GCT ATC ATC ACA ATG GAC NNN NNG 3'). Following the second strand synthesis the double stranded DNA can be amplified with a PCR or other nucleic acid amplification reaction (e.g. NASBA, TMA, rolling circle amplification).

In another embodiment the method used for the non-biased amplification of mRNA described above may be couple to

a transcription reaction by incorporation of a RNA polymerase promoter sequence in the primer used for the first strand synthesis. An example of such a primer would be:

5' AAT TCT AAT ACG ACT CAC TAT AGG $GT_{(10-30)}$ 3'

Following the second strand synthesis the double stranded DNA can be used as template in a PCR amplification and the amplified PCR fragments can be used as template in a transcription reaction with T7 RNA polymerase in case the primer described above is used that contains the T7 RNA polymerase promoter sequence. Following the second strand synthesis the double stranded DNA can also be directly used as template in a transcription reaction with T7 RNA polymerase in case the primer described above is used that contains the T7 RNA polymerase promoter sequence.

During the transcription reactions the RNA that is made can be labeled with a detectable moiety, e.g. a radioactive or fluorescent label. Subsequently the RNA can be used for analysis on microarrays or DNA chips for instance to elucidate the expression profile of the cells in the sample that the RNA was isolated from.

EXAMPLES

Materials and ingredients

All basic ingredients for buffers (Tris, $MgCl_2$, KCl, etc.) were purchased from Merck Nederland BV, Postbox 8198, 1005 AD Amsterdam, The Netherlands or Sigma-Aldrich Chemie BV, Stationsplein, Postbox 27, 3330 AA Zwijndrecht, The Netherlands. RNasin, MMLV reverse transcriptase and Amplitaq DNA polymerase were purchased from PE Applied Biosystems, Benelux, Hoogeveenenweg 100, Postbox 305, 2910 AH Nieuwerkerk a/d IJssel, The Netherlands. RNase H was purchased from Roche Diagnostics Nederland BV, Postbox 1007, 1300 BA Almere, The Netherlands. Sequenase DNA polymerase and dNTP's were purchased from Amersham Pharmacia Biotech, 800 Centennial Avenue, PO Box 1327, Piscataway, NJ 08855, USA. The TOPO-TA cloning kit (containing the pCR2.1-TOPO plasmid vector) was purchased from Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands Oligo's were purchased at different oligonucleotide suppliers and were usually purified by the supplier and tested for functionality in a PCR reaction with a known amount of input. Sequence analysis was performed using the ABI Prism kits purchased from PE Applied Biosystems, Benelux, Hoogeveenenweg 100, Postbox 305, 2910 AH Nieuwerkerk a/d IJssel, The Netherlands.

Input for a method of the invention is purified nucleic acid that can be isolated from (clinical) samples by a wide variety of methods. One of the methods that is very suitable for this purpose is the method described by Boom et al. (1990)[1].

Example 1

In this example we compared different primers to amplify MS2-phage RNA. Four different primers were tested (see table 1). After isolation and purification $4.0X\ 10^9$,

[1] *J Clin Microbiol* 1990 Mar;28(3):495-503 Rapid and simple method for purification of nucleic acids. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J

4.0X 10^7, 4.0X10^5, 4.0X10^3 and 4.0X10 copies of MS-2 phage RNA were used as input to do this experiment.

Table 1, Primers used in this example

Name	Sequence
JZH2R	5' GCT ATC ATC ACA ATG GAC NNN NNG 3'
JZH1	5' GCT ATC ATC ACA ATG GAC 3'

The experiment was performed using 10 μl of MS2 phage RNA at the amounts mentioned above. The RNA was heated 5 minutes to 80°C and subsequently cooled on ice to denature any double stranded nucleic acid and structures in the RNA. Subsequently, 10 μl of mixture A (100 mM KCl, 20 mM Tris, pH=8.3, 10 mM $MgCl_2$, 2 μM dATP, 2 μM dTTP, 2 μM dCTP, 2 μM dGTP, 50 ng primer [see table 1], 0.5 μl RNasin and 25 units MMLV reverse transcriptase) was added to the RNA and incubated for 10 minutes at ambient temperature, followed by an incubation at 42°C for 30 minutes. Subsequently the reaction was incubated at 80°C for 5 minutes and cooled down to ambient temperature followed by the addition of 0.5 μl RNase H (0.5 units) and further incubation at 37°C for 30 minutes. After this incubation the reactions are placed on ice to stop the reaction of RNase H.

The second strand synthesis was performed by using 20 μl of the first strand synthesis (on ice) and add 20 μl of mixture B (70 mM Tris, pH=7.5, 50 mM NaCl, 35 mM $MgCl_2$, 2 μM dATP, 2 μM dTTP, 2 μM dCTP, 2 μM dGTP, 100 ng primer with random sequence (see table 1) and 2.6 units Sequenase DNA polymerase) and incubate on ice for 10 minutes, subsequently incubate at ambient temperature for 10 minutes, followed by incubation at 37°C for 30 minutes. After the reaction the tubes are placed on ice and 2 μl is used for subsequent PCR amplification.

The PCR reactions were performed by adding to the 2 µl second strand synthesis reactions 48 µl PCR mix (50 mM Tris, pH=8,3, 20 mM KCl, 0.1 mg/ml BSA, 1.8 mM $MgCl_2$, 0.1 µM dATP, 0.1 µM dTTP, 0.1 µM dCTP, 0.1 µM dGTP, 100 ng PCR primer, see table 1) and incubate 95°C for 5 minutes followed by 45 cycles of 20 seconds 95°C, 30 seconds 55°C and 2 minutes 72°C. Following the last cycle the reactions are incubated for 10 minutes at 72°C, subsequently 10 minutes at 4°C and stored at 4°C or -20°C until further use.

In the PCR reactions primer JZH1 was used for PCR amplification of reactions that were made with primer JZH2R. In every experiment H_2O was used as negative control.

Of each PCR reaction 15 µl was run on an agarose gel and stained with ethidium bromide (standard protocols known to persons skilled in the art), the results are depicted in figure 2.

From the data in figure 2 it is clear that the JZH2R gives the best results with products visible on the agarose gel after PCR amplification when the method was started with only $4.0x\ 10^5$ copies MS2 phage RNA. Furthermore this example shows the applicability of the method to analyze RNA sequences.

Example 2

In this example we show the ability of a method of the invention to analyze DNA sequences. A Hepatitis B virus (HBV) positive serum was used as input to do this experiment. Nucleic acid isolated and purified from the serum were subjected to DNase I and RNase A treatment, respectively, to show the difference in analysis when either the DNA or RNA had been degraded. As a control a non-treated serum sample was analyzed. In addition to direct analysis of a serum sample the same set of experiments were done on the supernatants of a serum sample that was briefly centrifuged

(10 minutes at 3000 g) to remove any cellular debris in the serum sample.

The method used in this example was identical to the method used in example 1, with the following minor changes:

No RNasin was used for the analysis of DNA samples.

In case of a DNA template also a DNA dependent DNA polymerase may be used instead of the reverse transcriptase for the first strand synthesis. In this particular experiment reverse transcriptase was used.

The second strand synthesis was amplified 10 times in 10 independent PCR reactions.

The results are depicted in figure 3. The PCR fragments (see flowchart in figure 1) were analyzed on an agarose gel and visualized with ethidium bromide. Subsequently the bands from the gel were transferred to a filter with a standard blotting procedure, known to persons skilled in the art. The bands on the filter were interrogated (i.e. hybridized) with a specific digoxygenin (DIG) labeled probe covering the whole HBV genome of 3 kilobase (see figure 3)

The results clearly show that without any treatment of the serum sample (panel A in figure 3) the most bands in the 10 independent PCR reactions are observed. DNase treatment decreases the number of bands to virtually zero (panel B in figure 3), while RNase treatment still enables the amplification of some bands (panel C in figure 3). The DNase I treated nucleic acids contained no HBV positive bands (panel B, the right half of figure 3), while the RNase A treated nucleic acids still contained HBV positive bands confirmed by hybridized with Dig-labeled HBV probe bands (panel C, the right half of figure 3). This result demonstrate that this method can random amplify not only RNA as in example 1, but also DNA.

Example 3

In this example the serum of a patient suspected of infection with HIV-1 (antibody positive for HIV-1), HHV8, HBV and HGV was analyzed with a method of the invention. Nucleic acid was isolated and purified from 100 µl serum of this patient and 10 µl and 30 µl of nucleic acid solution (total 100 µl) was used for two independent experiments (the protocol as described in example 1 was applied). Per second strand synthesis (see flow chart in figure 1) 10 PCR reactions were performed. The PCR fragments were cloned in the TOPO-TA plasmid vector according to the manufacturer instructions (Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands). After transformation of *E. coli* cells a total of 198 different inserts in the plasmid were sequenced and of the 198 sequences 2 sequences were from HBV and 2 sequences were from HGV. The remaining 194 sequences were all of human origin or unknown (i.e. no homology found in the GenBank and EMBL nucleic acid databases). These results show that the method of the invention is capable of simultaneously analyzing both DNA (HBV genome) and RNA (HGV genome) sequences. The probable reason for not finding any HIV-1 or HHV8 sequences in the analysis in this example is most likely the low number of copies of the HIV-1 and HHV8 viruses in the sample used for the analysis.

Example 4

The same serum sample as used in example 3 was used in this example. We used 0.5 ml of serum to load on a 10 ml continuous sucrose gradient (10% to 60% sucrose w/v) which was centrifuged for 18 hours at 30.000 rpm at 4°C in a Beckman SW41Ti swing-out rotor in a Beckman ultracentrifuge. After the centrifugation 0.25 ml fractions were collected from the bottom of the tube. The density of the sucrose solution in each fraction was determined using a refractometer and each fraction was tested for the presence of HBV virus particles by a HBV specific PCR reaction

detection HBV DNA. The HBV DNA peak was found in fractions with a density of 1.186 g/ml to 1.205 g/ml. This peak fraction was pooled and nucleic acid isolated and purified. The nucleic acid was analyzed using the method as described in example 1. After the PCR reactions 14 different discrete bands were cloned in the TOPO-TA plasmid vector according to the manufacturer's instructions (Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands) and sequenced. All 14 inserts in the plasmid were HBV sequences and together they covered 2.8 kilobase of the HBV genome that is in total 3.2 kilobase.

A schematic presentation of the location of part of the sequences on the HBV genome is shown in figure 4. The data clearly show the applicability of the methods of the invention to obtain the nucleotide sequence of a long genome in a homogenous solution. The same method of course can be applied to the sequence analysis of long inserts in cloning systems like plasmids, phage lambda or yeast systems. The methods of the invention are very suitable for instance to determine the nucleotide sequences of inserts larger than 10 kilobase in phage lambda.

Example 5

The experiment in this example was performed with the protocol as described in example 1. The input material for the first strand synthesis was nucleic acid isolated from the culture supernatant of an HIV-1 culture. The primer that was used for the first strand synthesis was primer JZH2R (5' GCT ATC ATC ACA ATG GAC NNN NNG 3'). After the second strand synthesis (see example 1) the products were diluted in water and the serial dilutions used as input for the PCR amplification reaction. The PCR reaction (see example 1) was performed with primer JZH1 (5' GCT ATC ATC ACA ATG GAC 3'). Dilutions of 10, 100, 1.000, 10.000 and 100.000 times of the second strand synthesis were used for amplification.

The results are shown in figure 5. The results clearly show that input with a high complexity (i.e. many different sequences) will result in a smear of products after

the amplification illustrating the non-biased nature of the amplification. The complexity of sequences consists of cellular and viral sequences in the culture supernatant that is the result of cell lysis and non-adherent cells in the culture.

When the complexity of the input sequences is decreased by dilution the chances for a particular sequence of being part of the pool decreases and not all sequences are represented in the input material. Furthermore, the decrease in absolute copy number of the target sequences also allows only a few amplicons per target sequence to be made. Both these phenomena result in only a few bands per amplification (lanes 7-10). After cloning these bands are suited for sequence analysis, for instance of the HIV-1 genome. If the bands also contain human cellular sequences it may be necessary to purify the viral particles before application of the GAT procedure if the goal is sequence analysis of the HIV-1 genome. Such purification can be achieved by spinning down of cells and removal of background nucleic acid by DNase and RNase treatment prior to nucleic acid isolation. Dilution of the nucleic acid after second strand synthesis would than result in a few (10-100) bands to be the result of the amplification. These bands can than be sequenced showing the HIV-1 genomic sequence.

Example 6

First strand synthesis is performed as described in example 1 using total polyA mRNA isolated from cells as the input for the first strand synthesis (1-5 mg). The primer used for the first strand synthesis is GAT01 (5' *AAT TCT AAT ACG ACT CAC TAT AGG* GAG AGA AGG ATA CCA CTA GCT AGC GTT TTT TTT TTT TTT TTT TTT TTT T 3', the T7 promoter sequence is shown in *italics*). The second strand synthesis is performed as described in example 1 using JZH2R as primer (5' GCT ATC ATC ACA ATG GAC NNN NNG 3').

The second strand synthesis (or part thereof) is used as input for amplification in a NASBA reaction. The NASBA reaction ((Tris-HCl 40 mM, pH=8.5, $MgCl_2$ 12 mM, KCl 70 mM, DTT 5 mM, dNTP's (each) 1 mM, rATP 2 mM, rUTP 2 mM, rCTP 2 mM, rGTP 1.5 mM, ITP 0.5 mM, EDTA 0.75 mM, DMSO 15% v/v, oligonucleotide P1 [GAT02: 5' *AAT TCT AAT ACG ACT CAC TAT AGG* GAG AGA AGG ATA CCA CTA GCT AGC GT 3'], 0.2 µM, oligonucleotide P2 [JZH1: 5' GCT ATC ATC ACA ATG GAC 3'] 0.2 µM, and Sorbitol 0.375 M) was incubated at 65°C for 5 minutes and subsequently at 41°C for 5 minutes. Than the enzyme mix was added (BSA 2.1 mg, RNase H 0.01 units, T7 RNA Polymerase 37 units, AMV-RT 7.5 units) and after gentle mixing by tapping the reactions were incubated at 41°C in a water bath for 90 minutes.

The analysis of the amplification on an ethidium bromide stained agarose gel shows smears, indicating the non-biased amplification of all poly A mRNAs present in the input sample.

Example 7

The experiment in this example was performed with the protocol as described in example 1. The input material for the first strand synthesis was poly A+ mRNA provided as a control by the CLONTECH array systems. The amount of poly A+ mRNA used in subsequent reactions with diluted nucleic acid was equivalent to the amount of poly A+ mRNA present in 10.000, 1000, 100, 10 or 1 cell. First and second strand synthesis were performed as described in example 1. The complete cDNA product was subsequently used as input for the PCR amplification reaction. The PCR reaction (see example 1) was performed with primer JZH1 (5' GCT ATC ATC ACA ATG GAC 3') and after the amplification step the amplification products were purified using a NucleoSpin column, supplied with arrays (CLONTECH Inc., 1020 East Meadow Circle, Palo Alto, CA 94303, USA, www.clontech.com). Subsequently the amplification products were radiolabeled with alfa-32P-dATP

in a primer extension reaction with random hexamers or a collection of specific oligonucleotides supplied with the ATLAS arrays (CLONTECH Inc., 1020 East Meadow Circle, Palo Alto, CA 94303, USA, www.clontech.com).

The labeled products of the amplification were hybridized onto an ATLAS mouse array filter containing probes for approximately 600 genes of the mouse genome (CLONTECH Inc., 1020 East Meadow Circle, Palo Alto, CA 94303, USA, www.clontech.com). Hybridizations were performed according to the protocol CLONTECH supplies with the ATLAS arrays.

Results:

All dilutions of the poly A+ mRNA gave smears on gels, indicating the generic, non-biased amplification of all mRNA's in the sample. After hybridization of the amplified products generated with a poly A+ mRNA input equivalent to 1000 cells approximately 70 genes lighted up on the autoradiograph with some clear differences in the expression level of the genes. This result clearly shows the suitability of the GAT method described in this invention for preparing mRNA for analysis on (micro) arrays.

Brief description of the drawings

Figure 1. Schematic representation of one embodiment of the invention.

Figure 2. Ethidium bromide stained agarose gel showing the amplification products of the PCR reaction. Lanes 2-7: reactions performed with primer TAG7(N6). Lanes 9-13: reactions performed with primer TAG7(N5G); Lanes 14-19: reactions performed with primer TAG20; Lanes 21-26: reactions performed with primer JZH2R. Lanes 8 and 20 are markers. Input concentration of 4.0x 10^9 MS2 RNA copies was used for reactions loaded in lanes 2, 9, 14 and 21. Input concentration of 4.0x 10^7 MS2 RNA copies was used for reactions loaded in lanes 3, 10, 15 and 22. Input concentration of 4.0x 10^5 MS2 RNA copies was used for reactions loaded in lanes 4, 11, 16 and 23. Input concentration of 4.0x 10^3 MS2 RNA copies was used for reactions loaded in lanes 5, 12, 17 and 24. Input concentration of 40 MS2 RNA copies was used for reactions loaded in lanes 6, 13, 18 and 25. Negative control reactions are shown in lanes 7, 19 and 26.

Figure 3. Ethidium bromide stained agarose gel (left half) and HBV DIG-labeled probe hybridized blot (right half) of the 10 independent PCR reactions per second strand synthesis in this example. Panel A, non-treated serum; panel B, DNase I treated serum; panel C, RNase A treated serum. Lanes marked with M are marker lanes

Figure 4. Schematic presentation of the sequences obtained in this example (the arrows) on the HBV genome sequence (the red filled bar). The numbers in each arrow represent the lane number on the sequence gel (left to the slash) and the number of the clone (right of the slash).

Figure 5. Ethidium bromide stained agarose gel of the amplification results using a dilution of the second strand cDNA synthesis as input for the PCR amplification. Lane 1, 2 is 10-fold dilution, lane 3, 4 is 100-fold dilution, lane 5, 6 is 1.000-fold dilution, lane 7, 8 is 10.000-fold dilution, lane 9, 10 is 100.000-fold dilution.

Claims

1. A method for amplifying nucleic acids in a sample comprising:
-providing said sample with a set of primers comprising between 3-8 random bases and at least 8 essentially non-random bases,
-subjecting said sample to a first nucleic acid amplification reaction,
-providing said sample with at least one second primer comprising at least 8 bases essentially identical to said non-random bases,
-subjecting said sample to a second amplification reaction, and
-detecting nucleic acid amplified in said sample.

2. A method according to claim 1, wherein said set of primers comprises between 17-22 essentially non-random bases.

3. A method according to claim 1 or claim 2, wherein said set of primers comprises between 4-7 random bases.

4. A method according to anyone of claims 1-3 wherein said set of primers comprises 5 or 6 random bases.

5. A method according to anyone of claims 1-4, wherein said random bases are clustered at the 3' end of said set of primers.

6. A method according to anyone of claims 1-5, wherein said set of primers comprises a G at the 3' end.

7. A method according to anyone of claims 1-6, wherein said essentially non-random bases comprise a sequence enabling non nucleic acid primed nucleic acid synthesis.

8. A method according to claim 7, wherein said non nucleic acid primed nucleic acid synthesis comprises transcription.

9. A method according to anyone of claims 1-8, wherein said set of primers comprises the sequence,
- GCT ATC ATC ACA ATG GAC NNN NNG and/or
- AAT TCT AAT ACG ACT CAC TAT AGG GNN NNN G, wherein N can be any nucleotide or functional equivalent thereof.

10. A method according to anyone of claims 1-9, wherein said detecting of amplified nucleic acid comprises determining the sequence of at least part of said amplified nucleic acids.

11. A method according to anyone of claims 1-10, wherein said detecting of amplified nucleic acid comprises subjecting at least part of said amplified nucleic acids to a hybridization reaction with a multiplicity of nucleic acids preferably present in a microarray and/or DNA-chip and detecting whether amplified nucleic acids hybridized with one or more nucleic acids of said multiplicity of nucleic acids.

12. A method according to anyone of claims 1-11, further comprising an additional nucleic acid amplification of nucleic acids in said sample using at least one primer comprising essentially non random bases.

13. A set of oligonucleotides comprising a sequence
- GCT ATC ATC ACA ATG GAC NNN NNG and/or
- AAT TCT AAT ACG ACT CAC TAT AGG GNN NNN G, wherein N can be any nucleotide or functional equivalent thereof

14. Use of an oligonucleotide according to claim 13 for the preferred amplification of at least part of a viral nucleic acid.

15. Use of a set of primers comprising between 3 to 8 random bases clustered around the 3' end and one or more essentially

constant sequences clustered at essentially the 5' end of each primer in said set of primers, in a nucleic acid amplification reaction comprising nucleic acid, for providing complementary nucleic acid generated with said set of primers in said amplification reaction with one or more tags enabling further amplification and/or detection of said complementary nucleic acid.

16. A use according to claim 15, wherein said set of primers provide one essentially constant template for detection and/or further amplification of said complementary nucleic acid.

17. A kit for the amplification of nucleic acid in a sample comprising a set of primers comprising between 3-8 random bases.

18. A kit according to claim 17, wherein said nucleic acid comprises nucleic acid from a micro-organism or a derivative thereof.

19. A kit according to claim 16 or claim 17, comprising at least one set of oligonucleotides according to claim 13.

20. Use of a kit according to anyone of claims 17-19, in a method according to anyone of claims 1-12 or a use according to anyone of claims 14-16.

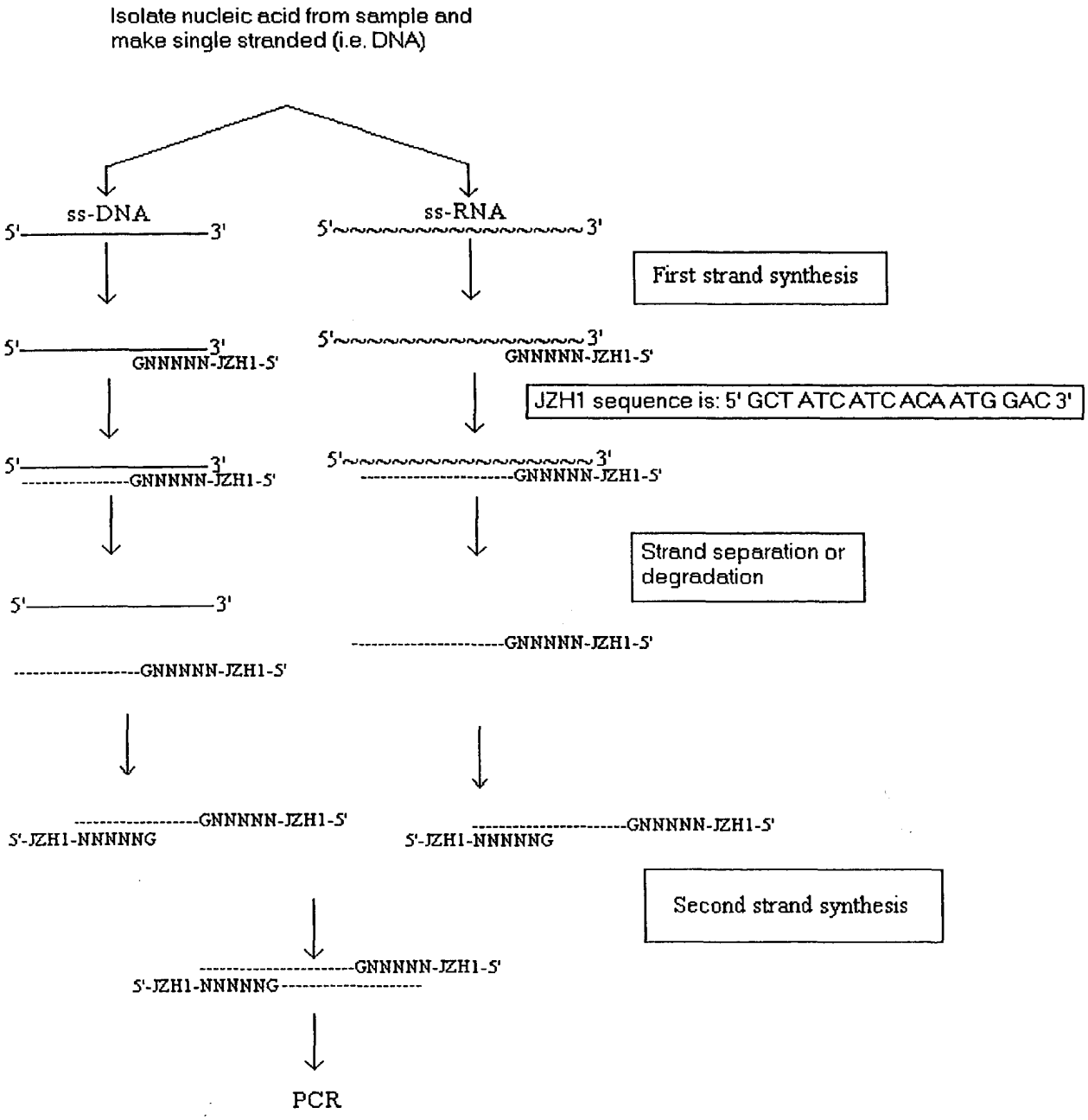

Isolate nucleic acid from sample and make single stranded (i.e. DNA)
ss-DNA
5'——3'
ss-RNA
5'~~~~3'
First strand synthesis
5'——3'
GNNNNN-JZH1-5'
5'~~~~3'
GNNNNN-JZH1-5'
JZH1 sequence is: 5' GCT ATC ATC ACA ATG GAC 3'
5'——3'
----------GNNNNN-JZH1-5'
5'~~~~3'
----------GNNNNN-JZH1-5'
Strand separation or degradation
5'——3'
----------GNNNNN-JZH1-5'
----------GNNNNN-JZH1-5'
----------GNNNNN-JZH1-5'
5'-JZH1-NNNNNG
----------GNNNNN-JZH1-5'
5'-JZH1-NNNNNG
Second strand synthesis
----------GNNNNN-JZH1-5'
5'-JZH1-NNNNNG----------
PCR

Fig. 1

Figure 2

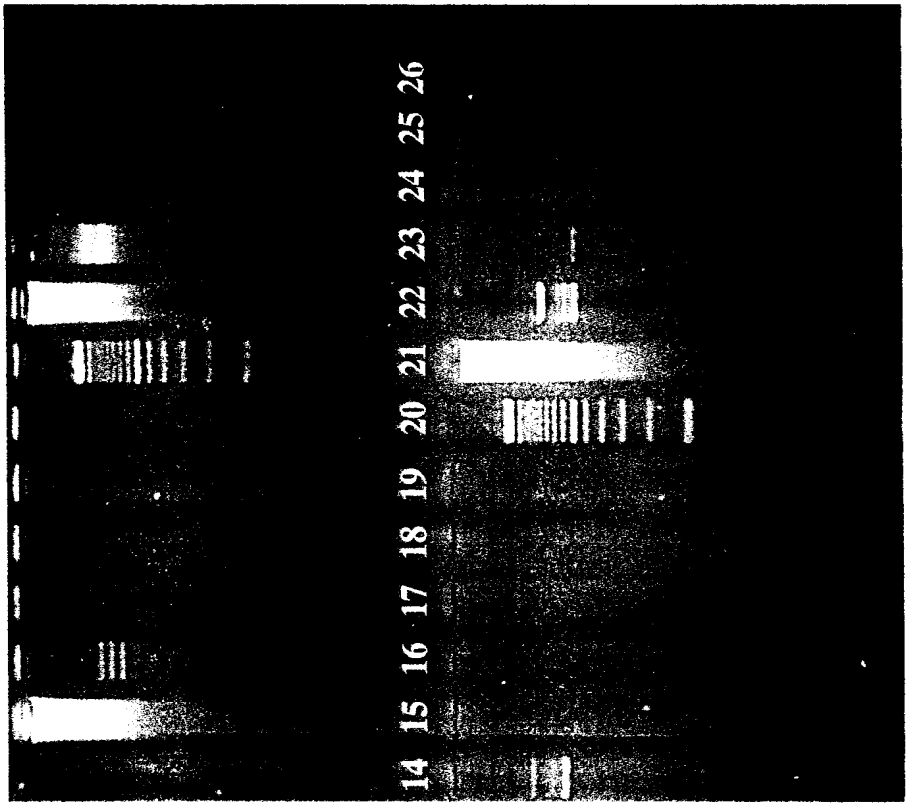
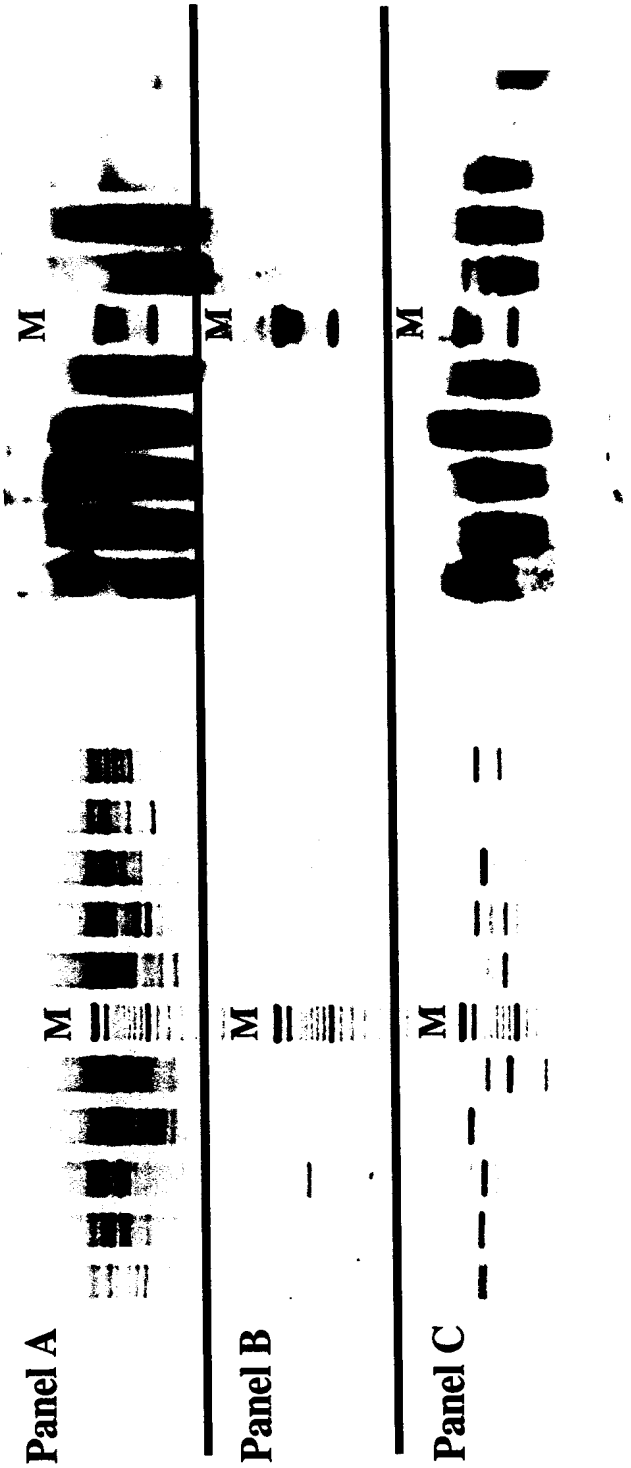


Figure 3



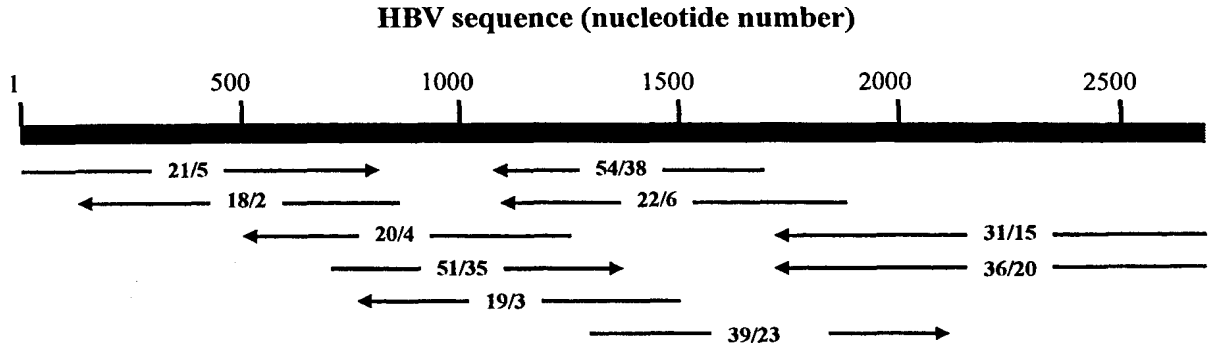

HBV sequence (nucleotide number)
1
500
1000
1500
2000
2500
21/5
54/38
18/2
22/6
20/4
31/15
51/35
36/20
19/3
39/23

Fig. 4

Figure 5